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13. ABSTRACT (Maximum 200 Words)
Prostate cancer is one of the most common malignant tumors with increasing incidence rates in the aging male, presenting a formidable public health problem. Gene therapy has been successfully exploited in several clinical trials. β-galactosidase(β-gal) as a reporter gene, has historically been the most popular gene for molecular biology work. A series expression *lacZ* gene vector was developed and two kinds of prostate cancer cells were screened by histology (X-Galstaining) and β-gal activity assay method to find highly expressing clones for the *lacZ* gene. Western blotting confirmed results. It will introduce a novel concept for further exploration for gene therapy using β-galactosidase to activate a broad-spectrum chemotherapeutic to assess the efficacy of the prodrugs *in vitro* and explore growth delay in animal models.

14. SUBJECT TERMS
Prostate cancer, β-galactosidase, gene therapy, PFONPG

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Introduction

Prostate cancer is one of the most common malignant tumors with increasing incidence rates in the aging male, presenting a formidable public health problem. Gene-based therapy has been stimulated by remarkable progress in understanding molecular biology. Gene therapy holds great promise for the treatment of diverse diseases, The *lacZ* gene, encoding the enzyme β-galactosidase (β-gal), has historically been the most common reporter gene used in molecular biology, many chromogenic or fluorogenic substrates are well established, but they are generally limited to histology or *in vitro* assays. A series expressing *lacZ* vector was develop and two kinds of prostate cancer cells highly express *lacZ* gene were screened by histology X-staining and β-gal activity assay method. Western blotting confirmed results. It will introduce a novel concept for further exploration for gene therapy using β-galactosidase to activate a broad-spectrum chemotherapeutic to assess the efficacy of the pro-drugs *in vitro* and explore growth delay in animal models.

There are three specific aims in my proposal:

Specific aim 1

Establish stable *lacZ*/*lacY* transfected CaP cell lines and screen the transfected cell colonies for high expression efficiency and growth capability. Months (1-12)

Specific aim 2

Evaluate cytotoxicity of pro-drug in transfected cells *in vitro*. (Months 12-18)

Specific aim 3

Evaluate cytotoxicity of pro-drug in transfected cells *in vivo*. (Months 18-24)

Body

Specific aim 1

Establish stable *lacZ/lacY* transfected CaP cell lines and screen the transfected cell colonies for high expression efficiency and growth capability.

Task 1. Develop a series of expression vectors for expressing *lacZ* and *lacZ/lacY* fusion open reading frame (Months 1-6)

Task 2. Transfect, screen and evaluate transfection efficiency and the optimal cell colonies for expressing β-galactosidase and lactose permease in CaP cells (MAT-Lu, PC3) (Months 3-9)

Task 3. Test lactose permease function in the transfected prostate cells (Months 6-12)

Task 4. Evaluate growth of cells *in vitro* and *in vivo* (Months 9-12)

Key Research Accomplishments

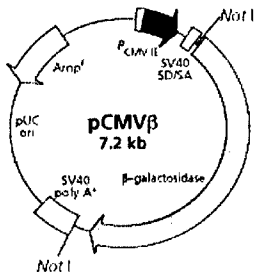
1. I developed a series highly expressing *lacZ* gene vectors
2. I screened two kinds of prostate cancer cells highly expressing *lacZ* gene

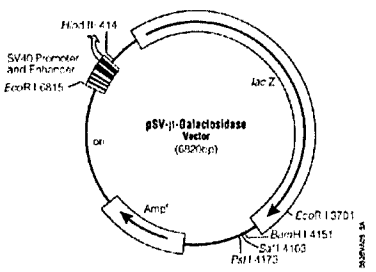
Key Research Accomplishments

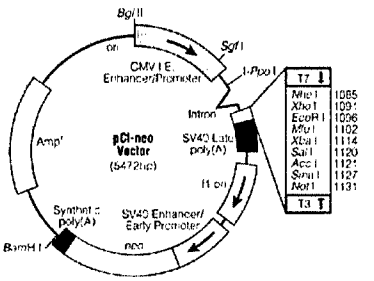
Task 1. Develop a series of expression vectors for expressing *lacZ* and *lacZ/lacY* fusion open reading frame (Months 1-6)

1. Commercial vectors

pCMVβ(*lacZ* gene, BD) or pSV-β-gal(*lacZ* gene, Promega) and pCI-Neo(N*eo* gene, Promega), *lacZ* and *Neo* gene are in different vectors, so they need to co-transfected to target cell.



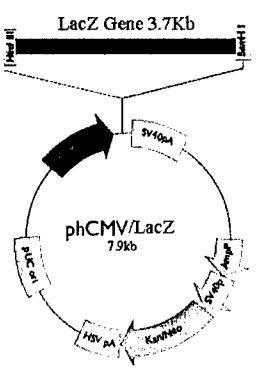


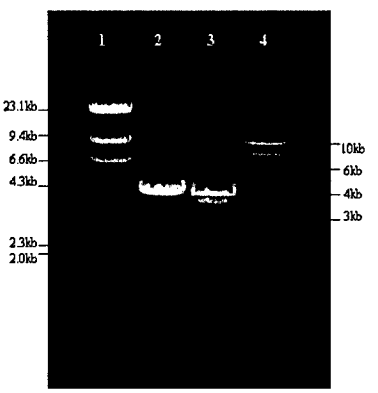


2. Developed two kinds of new high expression vectors

(1) phCMV/*lacZ*

Insert *lacZ* gene (include *lacY*, from pSV-β-gal vector, Promega) to high expression promoter vector phCMV (Gene Therapy Systems, Inc) to get recombinant vector phCMV/*lacZ*

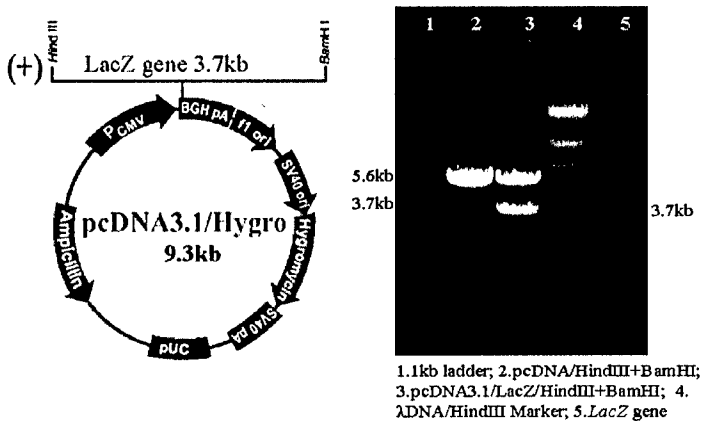




1. λ DNA/HindIII marker;
2. phCMV/HindIII+BamHI;
3. phCMV/lacZ/HindIII+BamHI;
4. 1 kb ladder

(2) pcDNA/3.1/Hyro/*lacZ*

Insert *lacZ*(include LacY) gene (from pSV-β-gal) to pcDNA3.1/hyro(Invitrogen) to get recombinant vector pcDNA/hyro/*lacZ*.

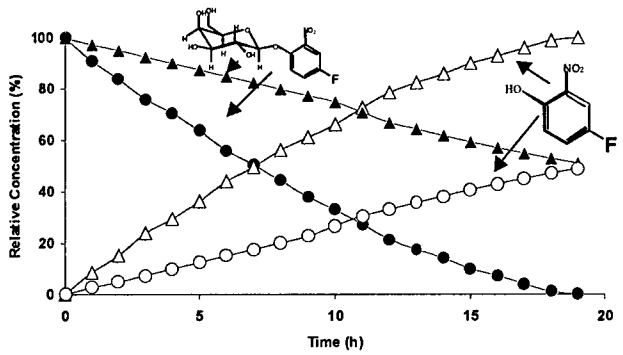


1.1kb ladder; 2.pcDNA/HindIII+BamHI; 3.pcDNA3.1/LacZ/HindIII+BamHI; 4. λDNA/HindIII Marker; 5.*LacZ* gene

Task 2. Transfect, screen and evaluate transfection efficiency and the optimal cell colonies for expressing β-galactosidase and lactose permease in CaP cells

1. Stable MAT-Lu/*lacZ* cell express β-gal

Using pCMVβ(*lacZ* gene, BD) or pSV-β-gal (Promega) and pCI-Neo (Promega, *neo* gene Co-transfected two plasmids to rat prostate cancer cell line Dunning prostate R3327 MAT-Lu which was selected by 600μg G418 in 10% FBS of DMEM. A few colonies were screened by detecting β-gal activity in OD_{420}, then selecting highest one and detect its β-gal expression by PFONPG (4-fluoro-2-nitrophenyl-β-D-galactopyranoside).

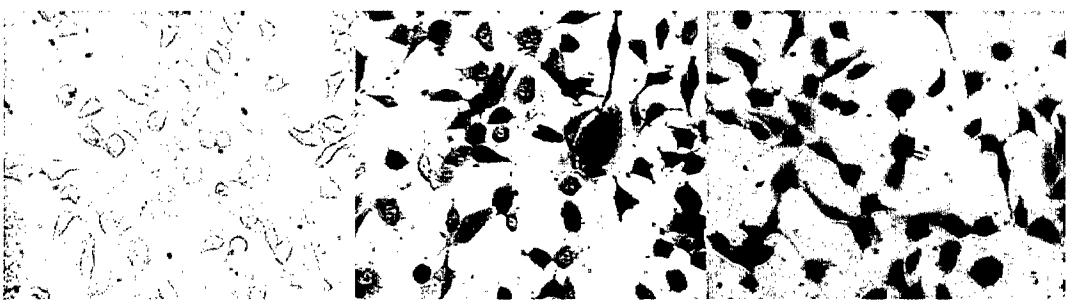


^{19}F NMR of the conversion of PFONPG (solid symbols) by stably transfected Dunning prostate R3327 MAT-Lu-*lacZ* cells (Δ 92×10^6) and MTLn3-*lacZ* (O 9.8×10^6) suspended in PBS at 37 °C.

This graph shows two stable cell lines by co-transfection express β-gal for hydrolyzing PFONPG.

2. Screened Stable PC3/*lacZ* cell

PC3 cells were maintained in the DMEM containing 10% FBS (Cellgro) and 20 mM glutamine at 37 ° C in an atmosphere of 95% humidified air and 5% CO_2. PC3 was transfected with phCMV/*lacZ* using GenePORTER2 (Gene Therapy Systems, Inc). PC3/*lacZ* cells expressing β-gal stably were selected by G418 (400 μg/ml; Cellgro). PC3 and PC3/*lacZ* cell, at about 60% confluency, were stained with the β-galactosidase staining kit in accordance with the manufacturer's instructions (Invitrogen). In brief, the growth medium was removed from the transfected cells (transfected by phCMV/*lacZ*) and the cells were rinsed once with PBS. The cells were fixed for 10 min at room temperature, rinsed twice with PBS, and then X-Gal staining solution and S-gal staining solution (+0.3mg/ml S-gal-Na and 0.3mg/ml FAC) were added to the cells for 6 h at 37° C. At least 200 cells were counted in four different fields (200x) and those cells that stained blue or black (for S-gal) were counted as expressing β-galactosidase; in PC3 cells transfected with a control plasmid (phCMV), no background staining was observed.



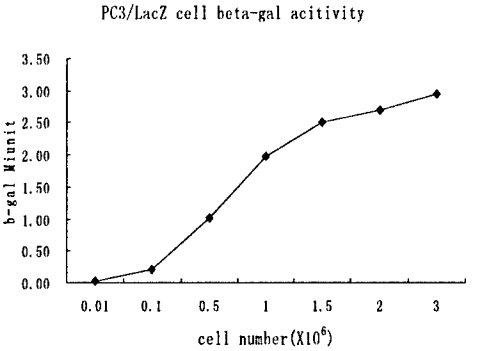
PC3 cells (X-gal, ×200) PC3/lacZ cell (X-gal, ×200) PC3/lacZ cell (S-Gal, ×200)

X-Gal and S-Gal stained the images showing PC3/*lacZ* cells, more than 90% PC3/*lacZ* cells were blue or black, the PC3 wild type cell was not color. This result shows the *lacZ* gene expression in PC3 after passage 30 generations.

Task 3. Test lactose permease function in the transfected prostate cells

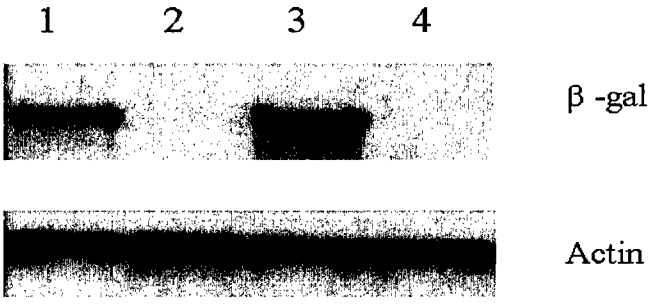
(1) PC3/*lacZ* cell β-gal activity assay

After the cells were counted and washed with PBS, they were lysed with 100 ml of report lysis buffer (Promega Corp., Madison, WI). Protein concentration was measured by using the protein assay kit (Bio-rad). β-gal activity was measured by spectroscopy (Bio-Rad) as described manual, and relative β-gal units were converted using recombinant β-galactosidase (Promega) as a standard. Duplicate measurements were performed for each concentration and three separate experiments were conducted; the data from the β-galactosidase activity was expressed as the definition of β-gal unit is that one unit will hydrolyze 1.0μmole of o-nitrophenyl β-**D**-galactoside to o-nitrophenol and D galactose per min.



(2). Western blotting detection of β-gal express in PC3 wild type and PC3/*lacZ* cells

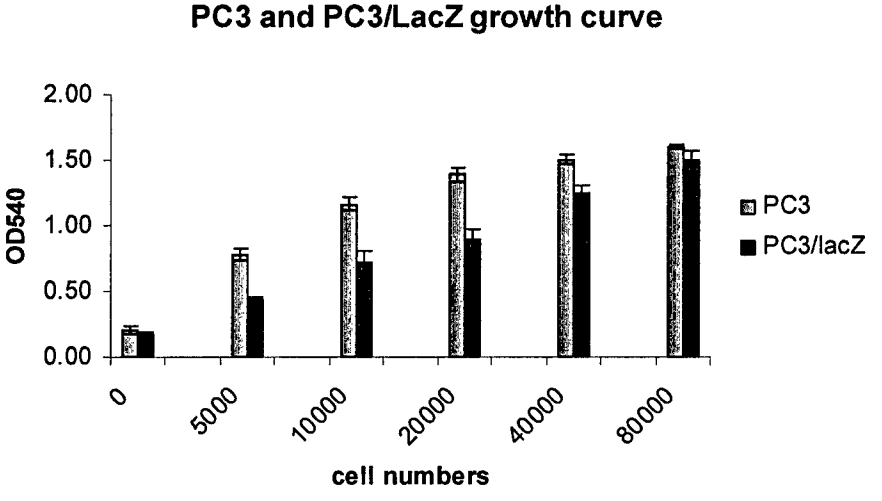
Detection of ß-galactosidase in Western blots. Total protein extraction for cells were electrophoresed, transferred to PVDF membrane and subsequently detected using mouse anti-ß-gal (Promega) and anti-Actin (Sigma) by HRP conjugated mouse anti-mouse IgG. Each blot was detected under the same conditions (30ug total protein) using Protein Detector Kit and treated with ECL substrate and exposed to film for 1 second. In PC3/*lacZ* cells, there is a clear band for β-gal (116kD) in PC3/*lacZ* cells (1,3), no signal was detected in PC3 cells (2,4).



Western blotting for PC3 WT and PC3/lacZ cell
1,3 PC3/*lacZ*; 2,4. PC3 WT

Task 4. Evaluate growth of cells *in vitro* and *in vivo*

1. Evaluate growth of cells *in vitro*
This graph shows that PC3 wild type cells grow a little faster than PC3/*lacZ* cells by crystal violet methods. According this result, we will detect the cytotoxicity on PFONPG *in vitro* and in *vivo*. For cell growth *in vivo* experiment, is on the way.



Reportable Outcomes

1. "In vivo detection of *lacZ* gene expression in a human prostate xenograft tumor by ^{19}F NMR using OFPNPG", **L. Liu**, V. Kodibagkar, J. Yu, **R. P. Mason**, Molecular Medicine Symposium, Houston, Feb. 2005

2. Detection of *lacZ* Gene Expression in MCF7 Rat Breast Tumor by Proton MRI, L. Liu, W. Cui, A. Adam, J. Yu, **R. P. Mason**, AACR 96th meeting, Alemeih, CA, Apr.2005.

3. "Novel Magnetic resonance Assays of Gene Imaging Constructs (MAGIC)" **R. P. Mason**, J. Yu, **L. Liu**, W. Cui and V. Kodibagkar, Molecular Medicine Symposium, Houston, Feb. 2005

4. Novel "Smart" ^{1}H MRI Contrast Agents for Assessing *lacZ* Gene Expression" J-X. Yu, **L. Liu,** V. D. Kodibagkar, W. Cui, R. D. Gerard, **R. P. Mason**, ISMRM 13th Scientific Meeting in Miami Beach, Florida, USA May 2005.

5. "^{19}F CSI of gene-reporter molecule OFPNPG", V. Kodibagkar, J. Yu, **L. Liu**, S. Brown, H. P. Hetherington, R. D. Gerard, and **R. P. Mason**, ISMRM 13th Scientific Meeting in Miami Beach, Florida, USA May 2005.

6. "Synthesis and Evaluation of a Novel Gene Reporter Molecule: Detection of β-galactosidase activity Using ^{19}F NMR of a Fluorinated Vitamin B_6 conjugate" J. Yu, Z. Ma, Y. Li, K. S. Koeneman, **L. Liu, R. P. Mason**, *Med. Chem.* in the press (2005)

7. "A Novel NMR Platform for Detecting Gene Transfection: Synthesis and Evaluation of Fluorinated Phenyl β-D-Galactosides with Potential Application for Assessing LacZ Gene Expression", J. Yu, P. Otten, Z. Ma, W. Cui, **L. Liu, R. P. Mason**, *Bioconjug. Chem.* 15 (6): 1334-1341 (2004)

8. "S-Gal^{TM}, a Novel ^{1}H MRI Reporter for β-Galactosidase", W. Cui, **L. Liu**, J. Yu, **R. P. Mason**, *Third Meeting of the Society for Molecular Imaging*, p200, St. Louis, MO, September 2004

9. Synthesis and Characterization of Novel Probes for *in vivo* Detection of *LacZ* Gene Expression, J.Yu, V. Kodibagkar, **L. Liu, R. P. Mason**, *Third Meeting of the Society for Molecular Imaging*, p200, St. Louis, MO, September 2004.

10. Magnetic resonance chemical shift imaging of gene-reporter molecule OFPNPG, V. Kodibagkar, J Yu, **L. Liu**, H. P. Hetherington and **R.P. Mason**, *Third Meeting of the Society for Molecular Imaging*, p200, St. Louis, MO, September 2004

Conclusions

1. I developed a series of recombinant *lacZ* gene expression vectors
2. Screened stable express *lacZ* gene cells, MAT-Lu/*lacZ* and PC3/*lacZ* cells
3. Confirm high expression of *lacZ* gene in these cells by beta-gal activity assay X-staining and West blotting.
4. Get the growth curve PC3 and PC3/*lacZ in vitro*.
5. I have also learnt techniques related to cell culture, tumor implantation and small animal handling and MRI.
6. While my goal is to achieve gene-activated toxicity, an analog agent, OFPNPG, low toxicity can be used as gene reporter molecular imaging. I have contributed to use, which has been accepted to publish as abstract based on my findings.

Appendices

In vivo detection of *lacZ* gene expression in a human prostate xenograft tumor By ^{19}F NMR CSI using OFPNPG

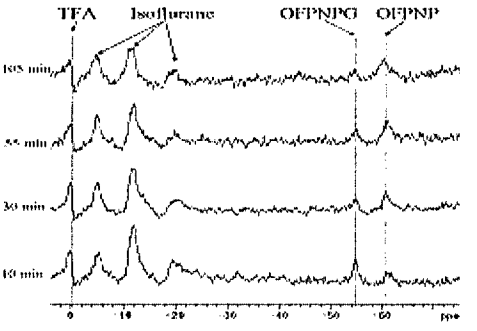
Li Liu, Vikram Kodibagkar, Jianxin Yu, Ralph P. Mason
Department of Radiology, The University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, Texas 75390-9058, USA. Email: Li.Liu@utsouthwestern.edu

Introduction

The *lacZ* gene, encoding the enzyme β-galactosidase (β-gal), has historically been the most common reporter gene used in molecular biology. Many chromogenic or fluorogenic substrates are well established, but they are generally limited to histology or *in vitro* assays. 2-Fluoro-4-nitrophenol-β-D-galactopyranoside (OFPNPG) belongs to a novel class of NMR active molecules (fluorophenyl-β-D-galactopyranosides), which are highly responsive to the action of β-gal. OFPNPG has a single ^{19}F NMR signal with chemical shift of 55 ppm. It is cleaved by β-gal to OFPNP, which has a pH sensitive chemical shift of 59-61 ppm. The large change in the chemical shift allows us to assess β-gal activity with magnetic resonance chemical shift imaging (CSI).

Methods

PC3/*lacZ* tumor cells were implanted in the thigh of SCID mice and allowed to grow to about 1 cm^3. When a solution of OFPNPG (4 mg in 50 μl water:DMSO::1:1 mixture with sodium trifluoroacetate (TFA) as a chemical shift reference standard) was injected intra-tumorally, signal was readily detected using a spin-echo CSI sequence at 4.7 T. Over a period of 2 h conversion of OFPNPG to product OFPNP was revealed by development of new upfield signal unequivocally demonstrating β-gal activity (see spectra)



Conclusion

These results provide our first observations in a tumor xenograft *in vivo* and show promise for the use of OFPNPG as gene-reporter molecule for future studies. Particular virtues of the NMR approach are the ability to detect specific substrate loss accompanied by product development unequivocally revealing enzyme activity. Simultaneously other metabolites can be observed here, the chemical shift standard TFA together with signals for the anesthetic isoflurane. As gene therapy becomes a reality, the ability to detect transgene expression non-invasively will become increasingly important for treatment planning and optimization.

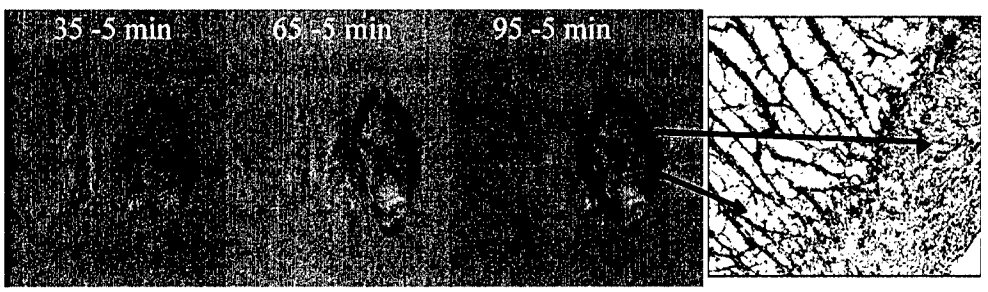
This research was supported in part by DOD PC031075 (LL) and NCI P20CA86354

Detection of *LacZ* Gene Expression in MCF7 Rat Breast Tumor by Proton MRI

Li Liu, Weina Cui, Ammar Adam, Jianxin Yu, Ralph P. Mason

Department of Radiology, The University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, Texas 75390-9058, USA. E-mail: Li.Liu@UTSouthwestern.edu

There is a pressing need for gene reporter molecules (GRMs), in particular, to facilitate magnetic resonance assay of gene expression. The lacZ gene, which codes β-galactosidase (β-gal) is one of the most widely used and best characterized reporter genes and diverse commercial substrates are available for histological analysis. S-gal is a new commercial "black stain" and we now demonstrate its use as a proton MRI contrast agent to selectively detect β-gal expression, revealed by strong T_2* contrast associated with the intensely paramagnetic black precipitate. No precipitate or contrasts were formed with wild type cells upon addition of S-gal and ferric ammonium citrate (FAC). In culture and *in vivo*, stably transfected MCF7-LacZ tumor cells rapidly showed development of paramagnetic precipitate. As an *in vivo* example, MCF7-LacZ cells were implanted in the thigh of a mouse and allowed to grow to about 1 cm diameter. S-gal-Na (50 mg/kg) and FAC (25 mg/kg) in saline were infused IP and MRI performed at 4.7 T over 2 h and subtraction images showed contrast development in the tumor periphery. Post mortem histology confirmed high β-gal activity in the tumor periphery with substantial necrosis in tumor center. We believe this novel approach based on Reporter Products for MRI (RPMs) holds great promise for imaging gene activity and detecting gene function *in vivo*.



Subtraction images over time from T_2* weighted gradient echo ^{1}H MRI (TR=500 ms, TE=8 ms, FOV= 256x256) of MCF-7/LacZ tumor. Contrast is primarily in tumor periphery. Post mortem histology showed intense stain in the tumor periphery with central tumor necrosis.

This research was supported by NCI P20CA86354 and DOD BrCa DAMD 17-03-1-0343-01 and Prostate (17-03-1-0101) Cancer Initiatives.

Novel Magnetic resonance Assays of Gene Imaging Constructs (MAGIC)

Ralph P. Mason, Jianxin Yu, Li Liu, Weina Cui and Vikram Kodibagkar,
Cancer Imaging Program, Department of Radiology, UTSouthwestern, Dallas

Gene-based therapy has been stimulated by remarkable progress in understanding molecular biology. Gene therapy holds great promise for the treatment of diverse diseases, but widespread implementation is hindered by difficulties in assessing the success of transfection: in particular, assessing the location, magnitude and persistence of transgene expression. Reporter genes and associated molecules should allow assessment of gene expression. Historically, the *lacZ* gene encoding the enzyme β-galactosidase (β-gal), has been the most common reporter gene used in molecular biology. Due to its broad spectrum of activity, many chromogenic or fluorogenic substrates are well established, but they are generally limited to histology or *in vitro* assays. We perceived novel approaches exploiting the virtues of magnetic resonance.

In one approach we have synthesized series of fluorophenyl-beta-D-galactopyranosides, which are direct analogs of the traditional "yellow' biochemical indicator ONPG (ortho-nitro-phenyl-galactopyranoside). The molecules show useful NMR characteristics with a single sharp ^{19}F NMR resonance, which is stable in whole blood and wild type tumor cells. In cells transfected with *lacZ* to express β-gal, there is rapid release of product accompanied by generation of a new well resolved signal. The range of analogs offers differential NMR and biological characteristics and we are currently optimizing this approach. Differences in chemical shift associated with small molecular variations allow multiple substrates to be interrogated simultaneously, potentially allowing several genes to be interrogated simultaneously. This methodology could provide a novel technology platform for diverse reporter genes and enzyme activities.

The ^{19}F NMR spectroscopy approach provides unequivocal evidence for gene activity, but proton MRI would have more immediate clinical application. We recently showed that the "black stain", S-Gal™ ((3,4-cyclohexenoesculetin-β-D-galactopyranoside)) has potential as a proton MRI reporter. Upon cleavage by β-gal in the presence of ferric ions, the aglycone produces an intense black stain, which is not only visible, but also paramagnetic yielding strong T_2* MRI contrast. We now report the first results *in vivo* demonstrating this novel approach to detecting gene activity in *lacZ* transfected MCF-7 tumor cells and MCF-7/*lacZ* xenograft tumor.

We believe the novel approaches based on Reporter Products for MRI (RPMs) hold great promise as a novel platform for imaging diverse gene activity and detecting gene function *in vivo*. More importantly, it could both accelerate the transfer of gene therapy to patients in the clinic and provide a new tool for the radiologist to evaluate gene therapy success.

Supported by the NCI Cancer Imaging Program (Pre-ICMIC P20 CA86354) and DOD Breast (DAMD 17-03-1-0343-01) and Prostate (17-03-1-0101) Cancer Initiatives.

^{19}F CSI of gene-reporter molecule OFPNPG

Vikram Kodibagkar[1], Jianxin Yu[1], Li Liu[1], Steven Brown[2], Hoby P. Hetherington[3], Robert Gerard[4], and Ralph P. Mason[1]

[1]Department of Radiology, The University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, Texas 75390-9058, USA.

[2]Henry Ford Health System, Detroit, USA.

[3]Department of Radiology, Albert Einstein College of Medicine, 1300 Morris Park Ave., Bronx, New York 10461, USA.

[4]Department of Internal Medicine and Molecular Biology, The University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, Texas 75390, USA.

Synopsis: The *lacZ* gene, encoding the enzyme β-galactosidase (β-gal) was historically the most attractive reporter gene for molecular biology. 2-Fluoro-4-nitrophenol-β-D-galactopyranoside (OFPNPG) belongs to a novel class of NMR active molecules (fluorophenyl-β-D-galactopyranosides), which are highly responsive to the action of β-gal. OFPNPG has a single ^{19}F peak with chemical shift of 55 ppm. It is cleaved by β-gal to OFPNP, which has a pH sensitive chemical shift of 59-61 ppm. The large change in the chemical shift allows us to image β-gal activity with magnetic resonance chemical shift imaging (CSI). We will present the results of ^{19}F CSI studies of enzyme activity and *lacZ* gene expression in 9L glioma and PC3 cells. Our results indicate that OFPNPG is a promising gene-reporter molecule for future *in vivo* studies.

Novel "Smart" 1H MRI Contrast Agents for Assessing LacZ Gene Expression

J-X. Yu[1], L. Liu[1], V. D. Kodibagkar[2], W. Cui[1], R. D. Gerard[3], R. P. Mason[1]
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Introduction

The application of reporter genes to study gene expression and regulation in biological systems is common practice. Among the widely used reporter proteins, β-gal (*lacZ*) is recognized as the most attractive reporter gene, and its introduction has become a standard means of assaying clonal insertion, transcriptional activation, protein expression, and protein interaction. Many colorimetric substrates are available commercially, but *in vivo* assays would be more powerful. Recently, Weissleder *et al.*[1] presented a near infrared *in vivo* approach based on DDAOG, Meade *et al.*[2] reported a proton MRI approach using EgadMe, and Mason *et al.*[3,4] presented both proton and ^{19}F NMR methods using S-gal™ and fluorophenol β-D-galactosides. S-gal™ was effective, but the molecule was designed for histology and can be optimized for *in vivo* MRI applications. We now present analogs of S-gal™ further demonstrating this fundamentally novel mechanism of "smart" ^{1}H MRI contrast agent, whereby the paramagnetic material is not generated until β-gal acts on the substrates (here AZD-3 or AZD-5) in the presence of Fe^{3+} ions to generate a precipitate (Figure 1).

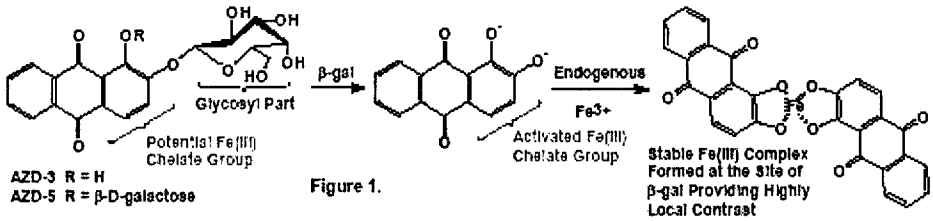


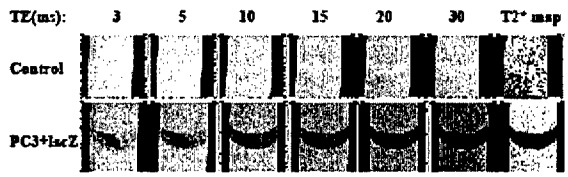
Figure 1.

Materials and Methods

AZD-3 and AZD-5 were stereoselectively synthesized and characterized in our lab. MR images were obtained using a Varian Unity INOVA 400 NMR spectrometer with gradient echo imaging: TR=100ms, Flip angle=10°, TE=multiple values 3-30ms, Matrix=256×128, FOV=48×24mm. As an example 10^5 PC3-LacZ or wild type cells were layered in agarose ferric ammonium citrate (2.5 μg/mL) and AZD-5 (1.5 μg/mL).

Results

A series of tests in solution and cultured tumor cells proved the principle. Both AZD-3 and AZD-5 were cleaved effectively by β-gal generating an intense black precipitate, which provides strong T_2^* relaxation and intense Fe(III)-based ^{1}H MRI contrast (Figure 2).



Conclusion

These results provide further evidence for the broad specificity of β-gal to cleave diverse substrates. The black paramagnetic precipitate is analogous to that formed using commercial S-gal™ and demonstrates the potential for derivatizing the substrate to optimize the MR active molecule. Here, ferric ions were added. However, it is noteworthy that tumor cells, as compared with their normal counterparts, frequently exhibit increased uptake and utilization of iron and thus endogenous ferric ions may suffice for *in vivo* applications. We believe, this novel "smart" Fe(III)-based ^{1}H MRI contrast agent mechanism holds great promise as a fundamentally different ^{1}H MRI platform for *in vivo* assessing *lacZ* gene activity.

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Novel NMR Platform for Detecting Gene Transfection: Synthesis and Evaluation of Fluorinated Phenyl β-D-Galactosides with Potential Application for Assessing LacZ Gene Expression†

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Gene therapy holds great promise for the treatment of diverse diseases, but widespread implementation is hindered by difficulties in assessing the success of transfection. The development of noninvasive reporter techniques based on appropriate molecules and imaging modalities may help to assay gene expression. Fluorophenyl-β-D-galactopyranosides provide a novel class of NMR active molecules, which are highly responsive to the action of β-galactosidase (β-gal), the product of the lacZ gene. The reporter molecules are stable in solution and with respect to wild-type cells, but the enzyme causes liberation of the aglycon, a fluorophenol, accompanied by distinct color formation and a ^{19}F NMR chemical shift of 5−10 ppm, depending on pH. Synthetic strategy, experimental methods, and molecular and ^{19}F NMR characteristics are reported for a series of molecules in solution, blood, and tumor cells. This class of molecules presents a new strategy for assaying gene expression with a highly versatile molecular structural platform.

INTRODUCTION

Gene therapy holds great promise for the treatment of diseases including cancer, cystic fibrosis, and immunodeficiency. However, a major hurdle to widespread successful implementation is the need to verify successful transfection, in particular, the spatial distribution of gene expression in the target tissue, together with assays of the longevity of expression. An image-based assay could greatly facilitate optimal gene therapy vector dosing, in a precise temporal and spatial manner.

Two approaches are gaining popularity for reporter genes. One method favors the use of genes producing reporter molecules such as green fluorescent protein, which are directly detectable by physical methods such as fluorescence. The second approach uses genes to produce enzymes, which act upon substrates administered to specifically interrogate gene expression. A critical criterion is that the reporter gene not be normally present or expressed in the cells of interest. The most popular reporter genes today are associated with optical imaging, because this is a cheap modality and is highly sensitive and the results are rapidly available (*1*, *2*). Thus, fluorescent imaging of green fluorescent protein [GFP[1] and longer wavelength variants (*3*)] and bioluminescent imaging (BLI) of luciferase activity on administered D-luciferin (*4*) are popular. These techniques are useful only in superficial tissues and have extensive applications in mice, but application to larger bodies is limited by shallow light penetration.

Several nuclear medicine approaches have been demonstrated by exploiting the action of thymidine kinase on a variety of substrates including iodo- and fluoronucleosides, such as FIAU and ganeyclovir, and various radionuclide labels including ^{123}I, ^{124}I, ^{125}I, and ^{18}F (*5*, *6*). For cancer, thymidine kinase has the advantage that not only does the gene serve as a reporter, but the gene products can themselves have therapeutic value. An alternative approach uses the sodium iodine symporter (hNIS), which works well with both iodide and pertechnetate substrates (*7*).

NMR has been applied to cells transfected to express melanin or transferrin resulting in iron accumulation, which produces proton MRI contrast (*8*, *9*). ^{19}F NMR has been used to detect conversion of 5-fluorocytosine to 5-fluorouracil following introduction of cytosine deaminase (*10*).

Historically, the bacterial *lacZ* gene, encoding the enzyme β-galactosidase (β-gal, EC 3.2.1.23), has been the most popular reporter gene. The lac operon was the first gene expression system to be well characterized, some 40 years ago by Jacob and Monod (*11*), and it is a recognized tool for the study of problems in cell and molecular biology and the recently emerging fields of genomics and proteomics (*12*). Its induction has become a standard means of assaying clonal insertion and transcriptional activation (*13*). The long-established tests for β-gal based on colorimetric assay of *o*- and *p*-nitrophenyl-β-galactopyranoside hydrolysis to release yellow *o*- or *p*-nitrophenols remain popular (*14*). However, because of the broad substrate specificity of the enzyme, alternate reporter substrates have been proposed (*15*−*19*), and many are commercially available. Fluorogenic galactosides based on fluorescein and resorufin, such as *p*-naphtholbenzein, 1,2-dihydroxyanthraquinone, 4-methylumbeliferone, 5-bromo-4-chloro-3-indoxyl-β-galactopyranoside (X-Gal), and 3,4-cyclohexenoesculetin-β-galactopyranoside (S-Gal) are well established (*16*, *20*−

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[1] Abbreviations: β-gal, β-galactosidase; BLI, bioluminescent imaging; GFP, green fluorescent protein; ONPG, *o*-nitrophenyl-β-galactopyranoside; PFONPG, 4-fluoro-2-nitrophenyl-β-D-galactopyranoside; S-Gal, 3,4-cyclohexenoesculetin-β-galactopyranoside; X-Gal, 5-bromo-4-chloro-3-indoxyl-β-galactopyranoside.

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Synthesis and Evaluation of a Novel Gene Reporter Molecule: Detection of β-galactosidase Activity Using ^{19}F NMR of a Fluorinated Vitamin B_6 Conjugate[+]

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Abstract: Gene therapy has emerged as a promising strategy for treatment of various diseases. However, widespread implementation is hampered by difficulties in assessing the success of transfection, in particular, the spatial extent of expression in the target tissue and the longevity of expression. Thus, the development of non-invasive reporter techniques based on appropriate molecules and imaging modalities may help to assay gene expression. We now report the design, synthesis and evaluation of a novel *in vivo* gene transfection reporter molecule 3-*O*-(β-D-galactopyranosyl)-6-fluoropyridoxol (GFPOL) using fluorinated vitamin B_6 as the ^{19}F NMR sensitive aglycone. GFPOL exhibits the following strengths as an *in vivo* ^{19}F NMR gene expression reporter: (a) large chemical shift response to enzyme cleavage ($\Delta\delta$=8.00 ppm); (b) minimal toxicity for substrate or aglycone; (c) good water solubility; (d) good blood stability; (e) pH responsiveness of aglycone.

Key Words: β-galactosidase, ^{19}F NMR, gene reporter, pyridoxol, pH.

INTRODUCTION

Gene therapy shows promise for the treatment various disorders and clinical trials are underway. However, non-invasive detection of transgenes *in vivo* would be of considerable value for assessing the location, magnitude and persistence of expression. Generally, therapeutic genes are not readily detected, and thus, various reporter genes have been developed and are widely applied in molecular biology, *e.g.*, β-galactosidase (β-gal), β-glucuronidase, chloramphenicol acetyltransferase, and firefly luciferase [1]. Among these, the *lacZ* gene, encoding β-gal, is the most attractive reporter gene, because β-gal activity is readily assessed *in vitro* in hosts as evolutionarily diverse as bacteria, yeast, and mammals, and its introduction has become a standard means of assaying clonal insertion, transcriptional activation, protein expression, and protein interaction [2]. Many chromogenic or fluorogenic substrates are well-established, but they are generally limited to histology or *in vitro* assays [3-8].

Recently, Weissleder *et al.* [9] presented a near infrared approach based on 9H-(1, 3-dichloro-9, 9-dimethylacridin-2-one-7-yl) β-D-galactopyranoside (DDAOG), and Meade et al. [10] reported an NMR approach using 1-[2-(β-D-galactopyranosyloxy) propyl]-4, 7, 10-tris (carboxymethyl)-1, 4, 7, 10-tetraazacyclododecane) gadolinium(III) (EgadMe), to assess β-gal activity *in vivo*. These diverse substrates emphasize the promiscuity (lack of substrate specificity) of β-gal activity. However, EgadMe was found to be 500 times less sensitive to β-gal than the traditional "yellow" biochemical indicator *ortho*-nitrophenol-β-D-galactopyranoside (*ONPG*) and failed to enter cells, necessitating direct microinjection. We realized that introduction of a fluorine atom into the traditional nitrophenol aglycones could generate NMR indicator molecules with minimal perturbation to a well-established molecular structure. Indeed, we successfully demonstrated the use of *p-fluoro-o*-nitrophenyl β-D-galactopyranoside (*PFONPG*) to detect enzyme activity in solution and transfected tumor cells [11]. *PFONPG* exhibits virtually identical sensitivity to cleavage by β-gal as compared with *ONPG* [12]. However, the liberated aglycone p-fluoro-o-nitrophenol (*PFONP*) exhibits some cytotoxicity, likely by analogy to the well known uncoupler of oxidative phosphorylation 2, 4-dinitrophenol [13]. More recently, we showed that various analogs of the aglycone structure (halophenols) showed significant differences in rate of response to enzyme action and some of these alternate aglycones exhibit much lower toxicity [12]. We now report the design, synthesis, and evaluation of another novel *in vivo* gene transfection reporter molecule using fluorinated vitamin B_6 as a stable aglycone and sensitive ^{19}F NMR indicator.

RESULTS AND DISCUSSION

Design

^{19}F NMR signals are exquisitely sensitive to molecular changes and often also to the microenvironment, and thus, there are many reporter molecules exploiting fluorine atoms [14]. We have previously shown that 6-fluoropyridoxol (**1**, **FPOL**) exhibits exceptional sensitivity to changes in pH

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S-Gal™, a novel ^{1}H MRI reporter for β-galactosidase

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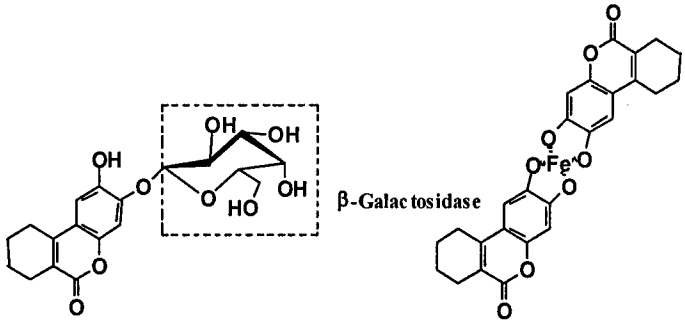
Gene-based therapy has been stimulated by remarkable progress in understanding molecular biology. However, progress and clinical trials would be enhanced by the ability to determine the location, the degree of activity and change in magnitude over time of the expression of the therapeutic genes. The development of non-invasive reporter techniques based on appropriate molecules and imaging modalities may help to assay gene expression. While many nuclear and optical imaging approaches have been presented as gene reporter methods, MR has lagged behind. LacZ, which produces β-galactosidase, has been the primary choice of reporter gene to verify effective transfection in biochemistry for many years, and due to its promiscuous activity many reporter molecules are available for biological and histological analysis. A recent addition to the detective toolkit is S-Gal™ (3,4-cyclohexenoesculetin-β-D-galactopyranoside). Upon cleavage by β-galactosidase in the presence of ferric ions (Fe^{3+}) he aglycone chelates iron to produce an intense black stain. In the spirit of multi modality approaches to imaging, it occurred to us that the iron (Fe^{3+}) complex is not only visible, but also paramagnetic. We will present data demonstrating this novel approach to detecting gene activity.



S-Gal™ is commercially available and readily enters cells. Action of β-gal rapidly generates an intense black precipitate, which induces strong T2 relaxation and intense MRI contrast. We believe this holds great promise as a novel MRI approach for imaging gene activity and detecting function.

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Synthesis and Characterization of Novel Probes for *in vivo* Detection of *LacZ* Gene Expression

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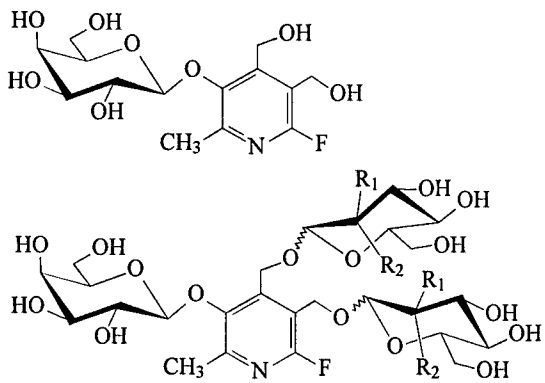
Gene therapy shows promise for the treatment various disorders and clinical trials are underway. However, It is thought that, non-invasive detection of transgene expression *in vivo* would be of considerable value for assessing the location, magnitude and persistence of transgene expression. Because the induction of *lac*Z gene encoding the enzyme β-galactosidase(β-gal) has become a standard means of assaying clonal insertion, transcriptional activation, protein expression, and protein interaction, *lacZ* gene has been the most attractive reporter gene for detection of β-gal activity. Many chromogenic or fluorogenic substrates are well-established, but they are generally limited to histology or *in vitro* assays. We recently demonstrated that introduction of a fluorine atom into the traditional biochemical substrate ortho-nitro-phenyl galactopyranoside, could provide a novel enzyme activity sensor (*viz*. gene reporter) with minimal perturbation to a well-proven substrate. We have now developed a novel approach incorporating the ^{19}F NMR pH reporter (6-FPOL). We will present the design, synthesis and evaluation of some new fluorinated vitamin B_6 β-D-galactosides as exciting substrates for *lacZ* gene expression using the ^{19}F chemical shift imaging.



R_1 = OH, R_2 = H β-D-Glucose
R_1 = H, R_2 = OH α-D-Mannose

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Magnetic resonance chemical shift imaging of gene-reporter molecule OFPNPG

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The *lacZ* gene, encoding the enzyme β-galactosidase (β-gal), has been the most attractive reporter gene for detection of β-gal activity. Many chromogenic or fluorogenic substrates are well-established, but they are generally limited to histology or *in vitro* assays. 2-Fluoro-4-nitrophenol-β-D-galactopyranoside (OFPNPG) belongs to a novel class of NMR active molecules (fluorophenyl-β-D-galactopyranosides), which are highly responsive to the action of β-gal. OFPNPG has a single ^{19}F peak with chemical shift of 55 ppm. It is cleaved by β-gal to OFPNP, which has a pH sensitive chemical shift of 59-61 ppm. The large change in the chemical shift allows us to image β-gal activity with magnetic resonance chemical shift imaging (CSI). We will present the results of ^{19}F CSI studies of enzyme activity and *lacZ* gene expression in 9L glioma cells. A standard spin-echo CSI sequence was used at 4.7 T for these studies. Sodium trifluoroacetate was used as a standard for quantifying dynamic changes on a voxel by voxel basis. Our results indicate that OFPNPG is a promising gene-reporter molecule for future *in vivo* studies.

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